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13. ABSTRACT (Maximum 200 Words)

Epidermal growth factor receptor (ErbB-1) and members of the ErbB family of receptor tyrosine kinases have been implicated in several mitogenic signaling pathways. Regulated growth factor-mediated signaling relies upon a balance between receptor activation, endocytosis, and degradation. As a result, overexpression or mutations altering receptor kinase activity disrupts this delicate balance, and is often sufficient to cause malignant transformation of the cell. We are particularly interested in the emerging role of the nonreceptor tyrosine kinase, ACK2, and its substrate, SH3PX1, in regulating the degradation of ErbB-2 family members. Establishing a role for ACK2 and SH3PX1 in ErbB-2 receptor degradation is especially appealing based on the predictive property between receptor overexpression and breast cancer. Currently, we are interested in further characterizing the ACK2-SH3PX1 interaction and determining the significance of ACK2-dependent phosphorylation of SH3PX1 in cells. To address these objectives, we have carried out deletion analysis studies to delineate the region of the phosphorylation site(s) on SH3PX1. In our studies, we have demonstrated that the phosphorylation signal of SH3PX1 is lost in the C-terminal truncation mutant ΔC395. In parallel site-directed mutagenesis studies, we conclude that all conserved point-mutants of SH3PX1 retain a phosphorylation signal comparable to *wild-type*. At this point, we believe that Mass Spectrometry may provide a more sensitive means to identify the ACK2 phsophorylation site on SH3PX1. Currently, efforts to generate recombinant forms of ACK2 and SH3PX1 are underway to aid in phosphopeptide mapping experiments, as well as screens for ACK2 inhibitors. The ability to regulate this phosphorylation event, perhaps through the identification of a dominant-negative form of SH3PX1, or specific inhibitors of ACK2, will help determine the importance of ACK2 activity in receptor endocytosis and degradation.

14. SUBJECT TERMS

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Introduction

Regulation of growth factor receptor expression and kinase activity is critical in several signal transduction pathways, including mitogenic pathways. The normal regulation of cell growth is achieved through a balance between the activation, endocytosis, and degradation of growth factor receptors. As a result, receptor overexpression or mutations altering receptor kinase activity can disrupt this delicate balance, and is often sufficient to cause malignant transformation of the cell. Of the various known growth factor receptors, the epidermal growth factor receptor (EGFR) and other ErbB family members are probably the best studied and most highly characterized. Overexpression of the ErbB family of receptors appears to play a causative role in various forms of cancer [1-6]. In particular, ErbB-2/Neu has received special attention in the clinical field based on the relationship between ErbB-2/Neu gene amplification and human breast cancers, with overexpression correlating with a poor prognosis for breast cancer patients [7,8].

Given the association of breast cancer with ErbB-2/Neu overexpression, much effort has been directed toward identifying therapeutic agents that will down-regulate ErbB-2/Neu activity. Currently, the use of monoclonal antibodies that inhibit ErbB-2/Neu activity, paired with chemotherapy, is the most successful mode of treatment for patients with metastatic breast cancer [9]. Preliminary results attribute the observed reduction in tumor growth to the acceleration of ErbB-2/Neu degradation, promoted by the monoclonal antibody, Trastuzumab (Herceptin^{TM}) [10]. Based on these findings, our studies are now focused on elucidating the molecular machinery underlying growth factor receptor endocytosis and degradation. To do so, we must identify the major proteins involved and develop tools to gain insight into this complex cellular process.

Two such proteins, ACK2 and its substrate, SH3PX1 have been linked to endocytosis and sorting through their ability to associate with various proteins involved in the processes and their effects on receptor degradation. More specifically, we and others have demonstrated that ACK2 and SH3PX1 form complexes with endocytic proteins, including clathrin, dynamin-2 and AP-2 [11,12,13]. In addition, overexpression of ACK2, SH3PX1, or various combinations of the two or their mutants, results in changes in the processing and trafficking of EGF and transferrin receptors [12,13].

ACK2 is a nonreceptor tyrosine kinase specifically activated by the Rho family GTP-binding protein, Cdc42. The domain structure of ACK2 consists of several signaling domains, including a tyrosine kinase domain, an SH3 domain, a CRIB (Cdc42/Rac interactive-binding) domain, two proline-rich domains, and a clathrin-binding domain [12,14]. SH3PX1 was identified as a binding partner and substrate for ACK2 through a series of GST pull-down assays [13]. SH3PX1 (sorting nexin 9, SNX9), a member of the nexin family of vesicle transport proteins, undergoes an EGF-dependent phosphorylation, mediated via the Cdc42-promoted activation of ACK2 [13]. While the precise role of SH3PX1 phosphorylation remains to be established, various lines of evidence point to a sorting function involving the EGF receptor and related family members. At least one other member of the nexin family, sorting nexin 1 (SNX1), has been implicated in EGF receptor sorting and degradation [15]. Sorting nexin 1 was found to bind the cytoplasmic domain of the EGF receptor through yeast two hybrid approaches, and has been implicated in directing EGF receptors to the lysosome for degradation [15]. Similarly, recent data from our laboratory suggests that the ACK2-catalyzed phosphorylation of SH3PX1 stimulates EGF receptor degradation [13].

Investigating the role of ACK2 and SH3PX1 in growth factor receptor degradation may lead to a better understanding of receptor overexpression and subsequent cellular transformation. Determining the significance of ACK2-dependent phosphorylation of SH3PX1 in cells, and developing the ability to regulate this phosphorylation event, perhaps through the identification of specific inhibitors of ACK2, will further our understanding of ACK2 activity in receptor endocytosis and degradation and its contribution to malignancy.

Progress Report

Task 1. Biochemical characterization of the interaction of ACK2 with its phospho-substrate, Months 1-12:

Deletion Mutant Analysis

To delineate the region on SH3PX1 that contains the phosphorylation site(s) for ACK2, a series of C-terminal truncation mutants were designed. Mutants ΔC84, ΔC197, ΔC339, ΔC395, ΔC547 were engineered with BamH1 and EcoR1 restriction sites and generated by PCR (PCR Sprint, Hybaid). The PCR products were directly ligated into the HA-tagged pcDNA3 expression vector using the Topo TA cloning kit from Invitrogen. Phosphorylation of these mutants was measured by co-expression of the ACK2 and SH3PX1 constructs in COS-7 cells, followed by immunoprecipitation and Western blotting using an anti-phosphotyrosine antibody. Loss of phosphorylation, as detected with HRP-conjugated 4G10 antibody (Upstate), occurs between ΔC197 and ΔC339 in an ACK2-dependent manner (Figure 1).

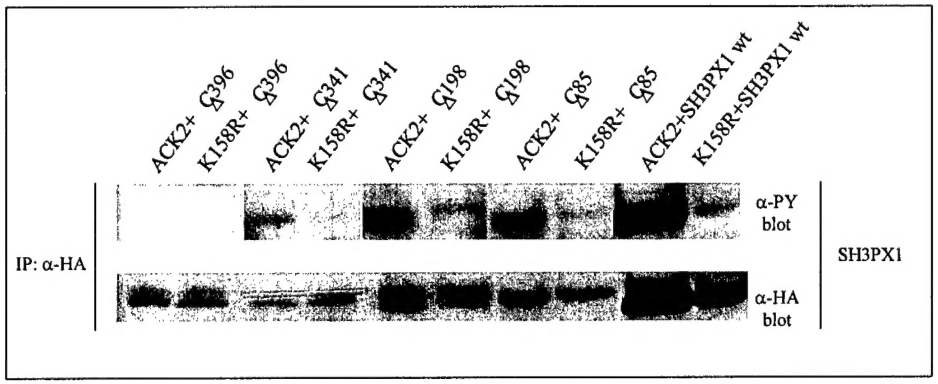

Figure 1. Phosphorylation of C-terminal Deletion Mutants of HA-SH3PX1
Deletion mutants were co-expressed with Myc-ACK2 in COS-7 cells. Deletion mutants were immunoprecipitated with anti-HA antibody from cell lysates and immunoblotted with anti-phosphotyrosine or anti-HA.

Point Mutant Analysis

Due to variable expression of the C-terminal truncation mutants and variable sensitivity of anti-phosphotyrosine antibodies, conserved tyrosine-to-phenylalanine point mutants were designed and loss of phosphorylation was measured in parallel through the afore-mentioned studies. Conserved mutants of *Drosophila* and human orthologs: Y9F, Y56F, Y287F, Y496F, Y546F, Y578F, and multiple mutants: F546/F578, F546/F561/F563/F578, and F546/F561/F563/F570/F578 were generated by PCR, using the Quick-change site-directed mutagenesis kit by Stratagene. Phosphorylation of the point mutants was carried out by co-expression of ACK2 and SH3PX1 mutants in COS-7 cells. Lysates were subject to immunoprecipitation, followed by immunoblotting with

anti-phosphotyrosine antibody. All single point mutants and mutiple point mutants retained a phosphorylation signal (Figure 2). Consequently, we believe that there may be multiple sites of phosphorylation on the substrate as we did not observe a decrease in phosphorylation of the mutants compared to *wild-type* SH3PX1. As a result, we believe that Mass Spectrometry may be a more sensitive method for determining the site of phosphorylation, and are in the process of generating recombinant kinase and substrate for the purpose of phosphopeptide mapping and the *in vitro* kinase screen (task 3).

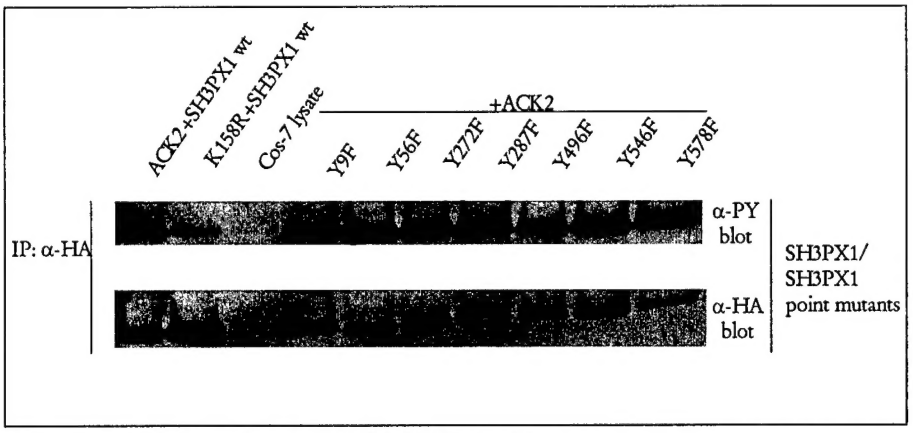

Figure 2. Phosphorylation of SH3PX1 Point Mutants
SH3PX1 point mutants were co-expressed with Myc-ACK2 in COS-7 cells. Point mutants were were immunoprecipitated with anti-HA antibody from cell lysates and immunoblotted with anti-phosphotyrosine or anti-HA.

Task 2. Evaluation of ACK2-dependent phosphorylation of SH3PX1 on the accumulation of receptors in breast cancer cells, Months 13-24:

To be initiated in fall 2004

Task 3. Combinatorial screen for ACK2 inhibitors/activators, Months 13-36:

Recombinant Protein Generation

Due to the size and complexity of ACK2 and SH3PX1, 83 kDa and 77 kDa, respectively, we initially set out to express these proteins in insect cells. His-tagged and untagged viruses of ACK2 and SH3PX1 were developed for expression of these proteins in Sf21 cells using the Invitrogen Bac-to-Bac kit. We soon found that SH3PX1, when expressed as a His-tagged protein in insect cells, retained a basal level of phosphorylation. We attributed this observation to the *Drosophila* orthologue of ACK2, DACK, and sought to eliminate this phosphorylation signal by co-expressing kinase-deficient ACK2-K158R with SH3PX1. However, we found that this had a minimal effect. Despite this set-back,

we were able to confirm ACK2 kinase activity from this host by co-infecting Sf21 cells and observing an increase in His-SH3PX1 phosphorylation by Western Blotting with anti-phosphotyrosine antibody (Figure 3).

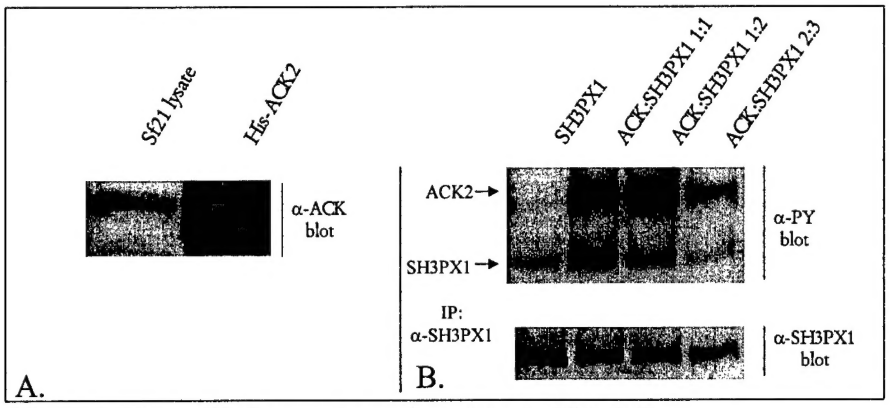

Figure 3. His-ACK2 Expression and Activity in Sf21 Cells
His-ACK2 expression in Sf21 cells measured by immunoblotting whole-cell lysates with anti-ACK antibody (panel A). ACK2 activity in Sf21 cells, carried out by co-infecting Sf21 cells with ACK2 and SH3PX1 viruses, varying the viral volume ratios, 1:1, 1:2, and 2:3 (ACK:SH3PX1, panel B). Detection of the phospho-substrate was carried out by immunoprecipitation with anti-SH3PX1 and immunoblotting with anti-phosphotyrosine and anti-SH3PX1.

To overcome the basal phosphorylation of our substrate, we expressed SH3PX1 as a GST fusion protein in *E. coli*, where the likelihood of phosphorylation was small. Full-length SH3PX1 was cloned into the pGEX-KG vector for recombinant expression. Expression of the GST-SH3PX1 fusion protein was carried out in the BL21 *E. coli* strain. One liter cultures were grown to an OD_{600} of 0.8 in super broth and induced with 200 μM IPTG overnight. Cells were harvested by centrifugation at 4000 rpm for 10 min in a JLA9.1 rotor (Beckman) and frozen at 80°C. All subsequent purification steps were carried out at 4°C. The bacterial pellet was resuspended in 1x TEDA (20 mM Tris [pH 7.9], 1 mM EDTA, 1 mM DTT, 1 mM NaN_3, 150 mM NaCl, 10% glycerol) supplemented with protease inhibitors (1 mM PMSF, 10 μg/mL each of aprotinin and leupeptin, 10 μM benzamidine) and lysed by three passages through a French Pressure Cell (SLM Aminco), followed by sonication for 5 minutes (550 Sonic Dismembrator, Fisher Scientific). The lysate was clarified by ultracentrifugation in a Ti45 rotor (Beckman) at 40,000 rpm. The clarified lysate was incubated for 30 min with glutathione-agarose beads (Sigma) pre-equilibrated in 1x TEDA. The GST-SH3PX1 fusion protein was then eluted with 50 mL of 10 mM glutathione in 1x TEDA and subsequently purified by size exclusion.
Large-scale preparations of His-ACK2 and GST-SH3PX1 are now underway.

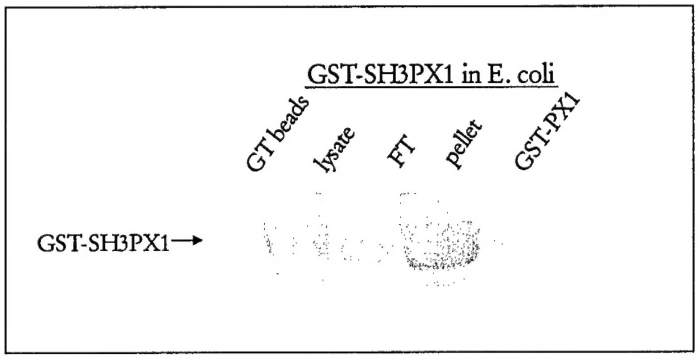

Figure 4. GST-SH3PX1 Expression in *E. coli*

Kinase Reaction

Preliminary kinase reactions have been performed incubating His-ACK2 with GST-PX1 on glutathione-agarose beads in 1x HMN (10 mM Hepes [pH 7.4], 5 mM $MgCl_2$, 150 mM NaCl) in the presence of 1 mM Na_3VO_4 and 1 mM ATP for 30 minutes at 30 degrees Celsius. The kinase reaction was quenched with 5X SDS loading buffer. Phosphorylation of GST-SH3PX1 was measured by immunoblotting with anti-phosphotyrosine antibody (Figure 5).

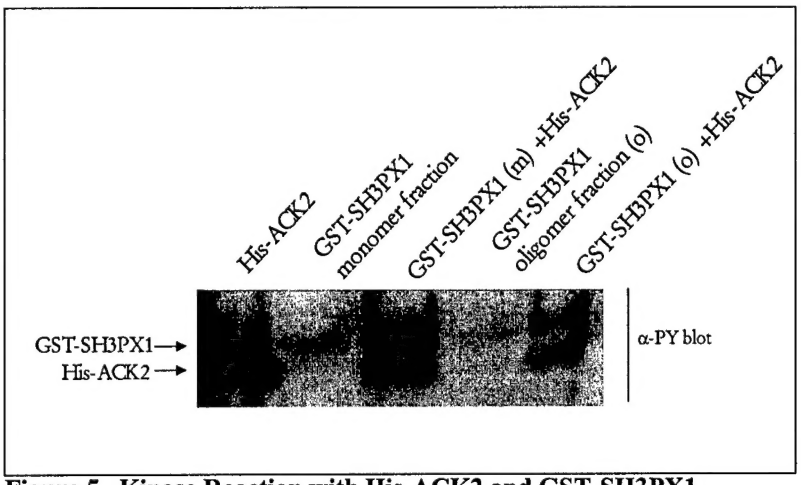

Figure 5. Kinase Reaction with His-ACK2 and GST-SH3PX1
GST-SH3PX1 exists in monomeric and oligomeric forms as determined by purification on a size exclusion column. GST-SH3PX1 samples from both fractions were incubated with His-ACK2 for 30 minutes at 30C. Both monomeric and oligomeric forms of GST-SH3PX1 appear to be phosphorylated as determined by Western blotting with anti-phosphotyrosine.

Screen for Inhibitors of ACK2

ACK2 kinase assays will involve fluorescence resonance energy transfer (FRET) detection systems, such as the Packard BioScience ALPHASCREEN™ or the LANCE™ detection system from Perkin Elmer. Both systems are based on energy transfer in the proximity of anti-phosphotyrosine antibody conjugated beads with phospho-substrate conjugated beads, to be read out by fluorescent detection. These reactions will be carried out in a 384-well or 1536-well microtiter plate, whereby multiple compounds will be screened in a facile manner.

An eight-week internship in the Automated Biotechnology Department of Merck was completed for the purpose of gaining experience in a high-throughput screening facility, and measuring the sensitivity of various fluorescence-based detection systems using insulin-like growth factor receptor (IGFR)-dependent phosphorylation of IGFR substrate. These approaches will be used to carry out a more rigorous screen for inhibitors of ACK2 phosphorylation activity in the coming months.

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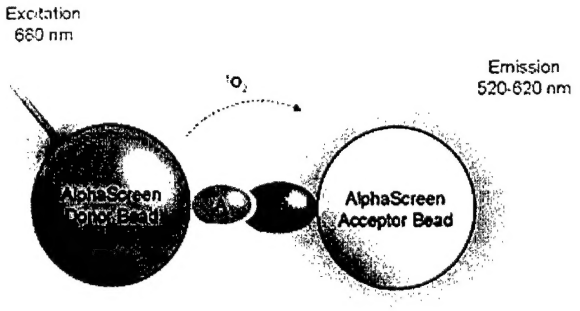

Research Accomplishments

- Generation and expression of C-terminal truncation mutants of SH3PX1—
  The loss of phosphorylation of SH3PX1 occurs between ΔC197 and ΔC339 in an ACK2-dependent manner, as detected by HRP-conjugated anti-phosphotyrosine antibody from Upstate.

- Generation and expression of SH3PX1 point mutants—
  All single, conserved point mutants retain a phosphorylation signal comparable to wild-type SH3PX1.

- Expression and purification of ACK2, kinase-deficient ACK2-K158R, and SH3PX1 viruses—
  1. ACK2 activity confirmed in insect (Sf21) cells
  2. Basal phosphorylation of SH3PX1 detected from Sf21 cells

- Expression, and Purification of GST-SH3PX1—
  GST-SH3PX1 is expressed in *E. coli* (BL21) cells and purified on glutathione-agarose beads

- Kinase Reaction—
  ACK2, purified from Sf21 cells, is able to phosphorylated GST-SH3PX1 on beads in an *in vitro* kinase assay

Reportable Outcomes

Characterization of the ACK2-SH3PX1 Interaction and its Role in Receptor Endocytosis
Carrie J. Stearns and Richard A. Cerione, Book of Abstracts, 43rd Annual ASCB Meeting, San Francisco, December 13-17, 2003

Conclusions

Investigating the role of ACK2 and SH3PX1 in growth factor receptor degradation may lead to a better understanding of the mechanisms underlying normal cell growth and proliferation and how these controls are disrupted in transformed cells. Based on the ties between overexpression of ACK2 and SH3PX1 and changes in the processing and trafficking of EGF and transferrin receptors, we are interested in further characterizing the ACK2-SH3PX1 interaction, and determining the significance of ACK2-dependent phosphorylation of SH3PX1 in cells. To date, we have demonstrated the loss of phosphorylation in the ΔC339 mutant of SH3PX1 by deletion analysis, and believe that there are multiple phosphorylation sites on the substrate due to mutagenesis studies. We hope to employ Mass Spectrometry in phosphopeptide mapping experiments to address this question. Improving our ability to regulate this phosphorylation event, by developing a dominant-negative form of SH3PX1 or by identifying specific inhibitors of ACK2, will further our understanding of ACK2 activity in receptor endocytosis and degradation and its contribution to malignancy.

References

1. Pavelic, K., Banjac, Z., Pavelic, J. & Spaventi, S. (1993) Evidence for a role of EGF receptor in the progression of human lung carinoma. *Anticancer res.*, **13**: 1133-1137.
2. Volm, M., Efferth, T. & Mattern, J. (1992) Oncoprotein (c-myc, c-erbB1, c-erbB2, c-fos) and suppressor gene product (p53) expression in squamous cell carcinomas of the lung. Clinical and biological correlations. *Anticancer res.*, **12**: 11-20.
3. Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. & Waterfield, M.D. (1984) Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. *Nature*, **307**: 521-527.
4. Libermann, T.A., Nusbaum, H.R., Razon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M.D., Ullrich, A. & Schlessinger, J. (1985) Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature*, **131**: 144-147.
5. Berger, M.S., Greenfield, C., Gullick, W.J., Haley, J., Downward, J., Neal, D.E., Harris, A.L. & Waterfield, M.D. (1987) Evaluation of epidermal growth factor receptors in bladder tumours. *Br. J. Cancer*, **56**: 533-537.
6. Blume-Jensen, P. & Hunter, T. (2001) Oncogenic kinase signalling. *Nature*, **411**: 355-365.
7. Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A. & McGuire, W.L. (1987) Human Breast Cancer: Correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science*, **235**: 177-182.
8. Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A. & Press, M.F. (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*, **244**: 707-712.
9. Baselga, J., Seidman, A.D., Rosen, P.P. & Norton, L. (1997) HER2 overexpression and paclitaxel sensitivity in breast cancer: therapeutic implications. *Oncology* **11**, 43-48.
10. Kasprzyk, P.G., Song, S.U., Di Fiore P.P. & King, C.R. (1992) Therapy of an animal model of human gastric cancer using a combination of anti-erbB-2 monoclonal antibodies. *Cancer Res.* **52**: 2771-2776.
11. Lundmark R., Carlsson S.R. (2003) Sorting nexin 9 participates in clathrin-mediated endocytosis through interactions with the core components. *J. Biol. Chem.*, **47**: 46772-46781.
12. Yang W., Lin Q., Zhao J., Guan J.L., Cerione R.A. (2001) The nonreceptor tyrosine kinase ACK2, a specific target for Cdc42 and a negative regulator of cell growth and focal adhesion complexes. *J. Biol. Chem.*, **276** (47): 43987-93.
13. Lin, Q., Lo, C.G., Cerione, R.A. & Yang, W. (2002) The Cdc42 target ACK2 interacts with sorting nexin 9 (SH3PX1) to regulate epidermal growth factor receptor degradation. *J Biol Chem.*, **277**: 10134-10138.
14. Yang, W. & Cerione, R.A. (1997) Cloning and characterization of a novel Cdc42-associated tyrosine kinase, ACK-2, from bovine brain. *J. Biol. Chem.*, **272**: 24819-24824.
15. Kurten, R.C., Cadena, D.L. & Gill, G.N. (1996) Enhanced degradation of EGF receptors by a sorting nexin, SNX1. *Science* **272**: 1008-1010.